

Synthesis and Antitumor Activity of New Benzoheterocyclic Derivatives of Distamycin A

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Received July 23, 1999

The design, synthesis, and in vivo and in vitro antileukemic activity of a novel series of compounds (13–22 and 34), in which different benzoheterocyclic rings, bearing a nitrogen mustard or a benzoyl nitrogen mustard or an α -bromoacryloyl group as alkylating moieties, are tethered to a distamycin frame, are reported, and structure–activity relationships are discussed. The new derivatives were prepared by coupling nitrogen mustard-substituted, benzoyl nitrogen mustard-substituted, or α -bromoacryloyl-substituted benzoheterocyclic carboxylic acids 23–32 with desformyldistamycin (33) or in one case with its two-pyrrole analogue 35. With very few exceptions, the activities of compounds bearing the same alkylating moiety are slightly affected by the kind of the heteroatom present on the benzoheterocyclic ring. All novel compounds, with one exception, showed in vitro activity against L1210 murine leukemia cell line comparable to or better than that of tallimustine. The compounds in which the nitrogen mustard and the α -bromoacryloyl moieties are directly linked to benzoheterocyclic ring showed potent cytotoxic activities (IC_{50} ranging from 2 to 14 nM), while benzoyl nitrogen mustard derivatives of benzoheterocycles showed reduced cytotoxic activities, and one compound (16) of this cluster was the sole derivative devoid of significant activity. Compound 18, a 5-nitrogen mustard *N*-methylindole derivative of distamycin, showed the best antileukemic activity in vivo, with a very long survival time (%T/C = 457), significantly increased in comparison to tallimustine (%T/C = 133), and was selected for further extensive evaluation. Arrested polymerase chain reaction and direct DNA fragmentation assays were performed for compound 18 and the structurally related compounds 13–17 and 19. The results obtained have shown that both alkylating groups and oligopeptide frames play a crucial role in the sequence selectivity of these compounds.

Introduction

There is currently interest in the study and development of low-molecular-weight sequence-selective agents interacting with double-stranded DNA. These molecules are often based on natural products and have been investigated for their ability to interact selectively with the minor groove of DNA. One of the most studied minor groove binders is distamycin A.

Distamycin A (1) is a naturally occurring antibiotic,¹ characterized by the presence of an oligopeptidic pyrrolecarbamoyl frame ending with an amidino moiety, which reversibly binds to the minor groove of DNA with a strong preference for adenine-thymine (AT)-rich sequences containing at least four AT base pairs.² Distamycin A has been used as a DNA sequence-selective carrier of alkylating functions, leading to compounds which are substantially more cytotoxic than distamycin A itself.

Mongelli and co-workers synthesized several semi-synthetic analogues of distamycin A wherein the formyl

group was replaced with different alkylating functions, such as benzoyl nitrogen mustard, nitrogen mustard, or α -bromoacryloyl moieties, obtaining the corresponding derivatives 2³ (tallimustine or FCE 24517), 3,⁴ and 4,³ respectively. Tallimustine (2) was selected as an antineoplastic drug candidate in view of its high activity against a wide spectrum of experimental tumors. However, since its clinical evaluation showed severe myelotoxicity, its development was discontinued.⁵

It is important to underline that, as occurs in the case of distamycin A and its four-pyrrole homologue 5, the increase in the number of pyrrole units of the oligopeptidic frame in 2–4 led to derivatives 6–8, respectively, which showed increased in vitro cytotoxicity and in vivo antitumor activity.^{3,4}

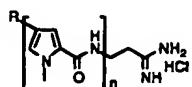
Recently, we have described⁶ the synthesis and the activity of two isosteric derivatives, 9 and 10, of 6 and 8, respectively, in which the *N*-methylpyrrole directly linked to the alkylating moiety was replaced by an *N*-methylpyrazole. Both these isosteres 9 and 10 showed antitumor activity against L1210 leukemia, which was almost equivalent to that exhibited by 6 and 8, respectively. It should be underlined that, on the contrary, the analogue 11 of compounds 6 and 9, in which a *N*-methylimidazole replaced the pyrrole or pyrazole unit, respectively, appeared about 2 orders of magnitude less

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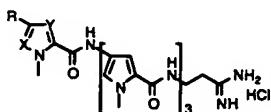
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Compound	n	R
1	3	NHCHO (Distamycin A)
2	3	4-[(C1CH2CH2)2N]C6H4CONH
3	3	(C1CH2CH2)2N
4	3	CH2=CBrCONH
5	4	NHCHO
6	4	4-[(C1CH2CH2)2N]C6H4CONH
7	4	(C1CH2CH2)2N
8	4	CH2=CBrCONH

cytotoxic than its pyrrole or pyrazole counterparts. Moreover, the analogue **12** of compounds **8** and **10**, in which a *N*-methylimidazole replaced the pyrrole or pyrazole unit, respectively, showed *in vitro* cytotoxicity against L1210 cells which was found to be equivalent to or slightly lower than that of **8** and **10**, the antileukemic activity *in vivo* being higher than that exhibited by **10**.



Compound	X	Y	R
9	N	CH	4-[(C1CH2CH2)2N]C6H4CONH
10	N	CH	CH2=CBrCONH
11	CH	N	4-[(C1CH2CH2)2N]C6H4CONH
12	CH	N	CH2=CBrCONH

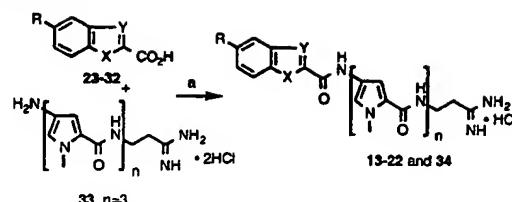
Following these results, we report in this paper the synthesis and the biological evaluation of the new distamycin A derivatives 13–22, in which the pyrrole ring bearing a nitrogen mustard, benzoyl nitrogen mustard, or α -bromoacryloyl alkylating moiety of compounds 6–8 has been replaced by different benzoheterocycles such as indole, *N*-methylindole, benzimidazole, and benzofuran.^{7a} Since pyrrole is susceptible to oxidative breakdown, these new derivatives have been prepared as potentially more stable minor groove binders aimed to improve the relative instability of the polypyrrolic skeleton. The choice of these benzoheterocycles can be also justified due to their importance in increasing the binding to DNA and the selectivity of alkylation of CC-1065 analogues such as U-71,184, adozelesin, and bizelesin.^{7b}

Chemistry

The synthetic route followed for the synthesis of derivatives 13–22 is outlined in Scheme 1. The key step was the coupling between benzoheterocyclic carboxylic acids 23–32 bearing the alkylating moieties and *N*-deformyldistamycin (33), obtained from distamycin A according to a reported procedure.^{1a} For the preparation of compound 34, the acid 31 was coupled with the known amino amidine 35.⁸

The condensations of the acylating agents 23–32 with deformyldistamycin (33) and of the acid 31 with 35 were

Scheme 1^a



⁸ Reagents: (a) EDC, Hunig's base, DME, 18 h, rt.

n	R	X	Y	Product
3	4-[(ClCH ₂ CH ₂) ₂ N]C ₆ H ₄ CONH	NH	CH	13
3	4-[(ClCH ₂ CH ₂) ₂ N]C ₆ H ₄ CONH	NCH ₃	CH	14
3	4-[(ClCH ₂ CH ₂) ₂ N]C ₆ H ₄ CONH	O	CH	15
3	4-[(ClCH ₂ CH ₂) ₂ N]C ₆ H ₄ CONH	NH	N	16
3	(ClCH ₂ CH ₂) ₂ N	NH	CH	17
3	(ClCH ₂ CH ₂) ₂ N	NCH ₃	CH	18
3	(ClCH ₂ CH ₂) ₂ N	O	CH	19
3	CH ₂ =CBrCONH	NH	CH	20
3	CH ₂ =CBrCONH	NCH ₃	CH	21
3	CH ₂ =CBrCONH	O	CH	22
2	CH ₂ =CBrCONH	NCH ₃	CH	24

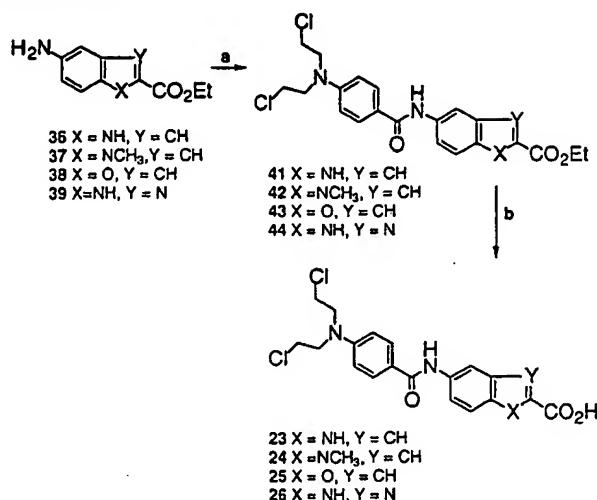
^a Reagents: (a) EDC, Hunig's base, DMF, 18 h, rt.

performed using a slight excess (1.5 equiv) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) as coupling agent, in dry DMF as solvent, in the presence of Hunig's base, at room temperature and with identical reaction times (18 h). Compounds **13–22** and **34** were prepared in acceptable yields, after purification by silica gel flash chromatography.

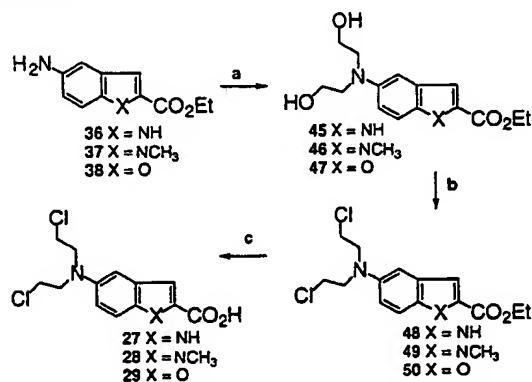
The synthesis of heterocyclic acids 23–26, bearing the benzoic acid mustard (BAM), was performed by coupling ethyl 5-aminoindole-2-carboxylate (36),⁹ ethyl 1-methyl-4-aminoindole-2-carboxylate (37),¹⁰ ethyl 5-aminobenzo[furan-2-carboxylate (38), and ethyl 5-amino-1(3H)-benzimidazole-2-carboxylate (39), respectively, to *p*-[bis(2-chloroethyl)amino]benzoyl chloride (40)¹¹ to give the corresponding amido esters 41–44, which after purification by flash chromatography were submitted to mild alkaline hydrolysis to yield the corresponding acids 23–26, respectively (Scheme 2).

For the synthesis of acid intermediates 27–29, the amino esters 36–38 were converted in good yields to the corresponding *N,N*-bis(2-hydroxyethyl) derivatives 45–47, respectively, by reaction with a large excess (5 equiv) of ethylene oxide in methanol. Subsequent treatment with phosphorus oxytrichloride (POCl_3) afforded the corresponding dichloro nitrogen mustards 48–50, which were transformed into the desired acids 27–29 by acid hydrolysis (Scheme 3).

The synthetic approach employed for the preparation of the α -bromoacryloyl derivatives 30–32 shown in Scheme 4 was carried out by conversion of the nitro carboxylic acids 51,¹² 52,¹⁰ and 53¹³ into the corresponding *tert*-butyl esters 54–56 in good yield by treating 52

Scheme 2^a

^a Reagents: (a) 40, Et₃N, CH₂Cl₂, 18 h, rt; (b) KOH, water/dioxane, 3 h, rt.

Scheme 3^a

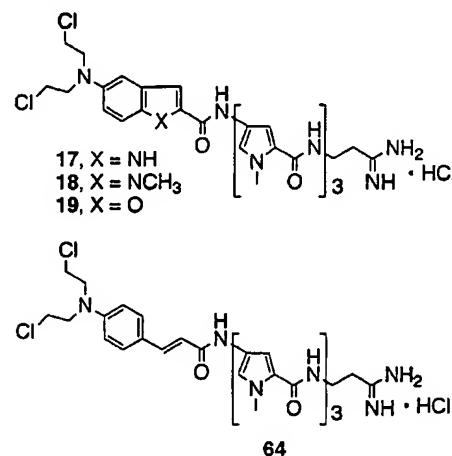
^a Reagents: (a) ethylene oxide; (b) POCl₃, toluene, rt, 1 h; (c) 37% HCl in water, rt, 3 h.

and 53 with carbonyldiimidazole (CDI) and then with *tert*-butyl alcohol¹⁴ and 51 with *tert*-butyl bromide¹⁵ (Scheme 4). The amino compounds 57–59, obtained by catalytic hydrogenation (10% Pd–C) of the corresponding nitro *tert*-butyl esters 54–56, after treatment with the α -bromoacrylic acid in the presence of 2 equiv of EDC afforded 60–62, respectively. Subsequent treatment with trifluoroacetic acid (TFA) at room temperature afforded the carboxylic acids 30–32.

Results and Discussion

In Vitro and in Vivo Antitumor Activity. The in vitro cytotoxic activity of the synthesized compounds 13–22 and 34 was evaluated against the L1210 murine leukemia cell line and in L1210 sublines resistant to tallimustine (L1210/tallimustine) and to doxorubicin (L1210/DX). The cytotoxic activity of the synthesized compounds has been compared with that of distamycin A (1), tallimustine (2), and the distamycin derivatives 3–12 and 64. This latter compound is a previously reported cinnamoyl nitrogen mustard derivative¹⁶ of distamycin A, a vinylogue of tallimustine. Compounds 17–19 should be considered as “conformationally constrained” forms of the cinnamic mustard derivative 64 of distamycin A, where the benzoheterocyclic unit

incorporating both the phenyl and the vinylic double bond of the cinnamic moiety confers rigidity to the alkylating region of the molecule.

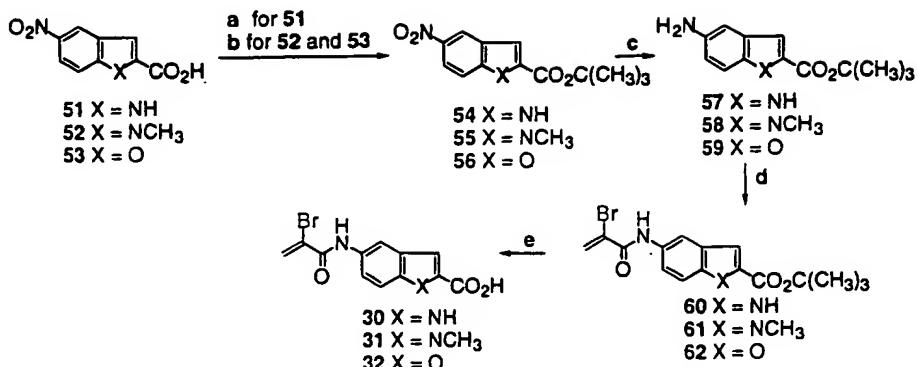


The compounds 13–22 and 34 were also tested in vivo against L1210 murine leukemia cells, and some representative molecules (18 and 21) were also tested on M5076 murine reticulosarcoma. The results obtained are summarized in Table 1.

Derivatives 13–15 containing the BAM as alkylating moiety showed significant cytotoxicity against L1210 cell line with IC₅₀ values comparable to that reported for tallimustine (2) but lower than those reported for both its pyrrole⁶ and pyrazole⁹ homologues. Derivatives 13–15 were at least 30-fold more cytotoxic than the benzoimidazole derivative 16, which showed anti-proliferative activity similar to that reported for the imidazole distamycin analogue 11.⁶ The poor activity of 16 may be related to a different DNA recognition sequence, due to the possible role of the N(3) lone pair of the benzoimidazole ring¹⁷ which may be capable to accommodate hydrogen bonding with the guanine 2-amino group and thus provide for GC selectivity. This could alter the strict preference for AT sequence allowing GC recognition and then mixed AT/GC sequences.

Compounds 13–15 not only exhibited good activity in *in vitro* experiments but also showed a significant increase in survival time (%T/C) of mice bearing the lymphocytic leukemia model (L1210). Derivatives 14 and 15 were 8- and 2-fold less potent than tallimustine (optimal nontoxic dose (OD): OD = 25 mg/kg for 14, 6.25 mg/kg for 15 vs OD = 3.13 mg/kg for tallimustine), while 13 showed a potency similar to that of tallimustine, with a median survival time slightly higher (%T/C = 163 for 13 vs %T/C = 133 for tallimustine). In this series of BAM tetrapeptides 13–15, the compound 14, which was the less potent in vivo, possessed a %T/C value comparable to that of tallimustine (%T/C = 183 for 13 vs %T/C = 133), while 15 showed a lower value (%T/C = 150). Moreover, compounds 13–15 were 2–4-fold less cytotoxic than the pyrrole 6 and pyrazole 9 counterparts.

The nature of the alkylating group had a great effect on the cytotoxicity of compounds having the same oligopeptide skeleton. In fact, the presence of a nitrogen mustard moiety directly linked to the benzoheterocyclic ring, as in the case of compounds 17–19, instead of a nitrogen benzoyl mustard moiety, as in the case of

Scheme 4^a

^a Reagents: (a) $(\text{CH}_3)_3\text{C-Br}$, K_2CO_3 , BTEAC, DMF; (b) CDI, *tert*-butyl alcohol, DBU, DMF; (c) H_2 , Pd-C; (d) α -bromoacrylic acid, EDCI, DMF; (e) $\text{CF}_3\text{CO}_2\text{H}$, rt, 1 h.

Table 1. In Vivo and in Vitro Activity of Distamycin Derivatives against L1210 Murine Leukemia^a

compd	in vitro L1210		in vivo L1210		
	IC_{50} (nM \pm SE)	ip	OD (mg/kg)	%T/C	OD (mg/kg)
distamycin A ³	10069 ± 1647	200	113	nd	nd
tallimustine (2) ³	68.5 ± 6.6	3.1	175	3.1	133
3 ⁴	3.9 ± 0.6	1.6	138	0.78	117
4 ³	79.6 ± 14	12.5	175	nd	nd
5 ³	445 ± 75	nd	nd	nd	nd
6 ³	19.0 ± 0.6	0.4	138	nd	nd
7 ⁴	0.081 ± 0.01	0.39	188	0.39	133
8 ³	6.3 ± 1.34	3.1	725	1.6	200
9 ⁶	34.5 ± 4.6	3.1	144	nd	nd
10 ⁶	13.3 ± 0.54	6.2	200	nd	nd
11 ⁶	2372 ± 157	6.2	125	nd	nd
12 ⁶	46.9 ± 13	6.2	163	nd	nd
13	95.3 ± 28	nd	nd	3.1	163
14	61.7 ± 2.49	nd	nd	25	183
15	43.5 ± 7	nd	nd	6.2	150
16	1422 ± 235	nd	nd	nd	nd
17	8.0 ± 2.6	nd	nd	0.39	174
18	14.7 ± 2.4	nd	nd	1.6	457
19	3.7 ± 1.5	nd	nd	0.39	200
20	4.1 ± 1.3	nd	nd	12.5	213
21	2.4 ± 0.39	nd	nd	6.2	192
22	6.1 ± 0.93	nd	nd	0.78	117
34	15.3 ± 4.31	nd	nd	3.1	133
64 ¹⁶	9.5 ± 2.71	nd	nd	6.2	267

^a IC_{50} = 50% inhibitory concentration as the mean \pm SE from dose-response curves of at least three experiments; ip = treatment was performed intraperitoneally on day 1 after tumor ip transplant; iv = treatment was performed intravenously on day 1 after tumor iv transplant; OD = optimal dose, optimal nontoxic dose $<$ LD_{10} (weight loss $<$ 20% with respect to the starting weight); %T/C = median survival time of treated vs untreated mice \times 100; nd = not determined.

compounds 13–16, proved to be effective in increasing the cytotoxic activity by a factor of up to 10.

Especially noteworthy was the compound 18, which although 2- or 4-fold less active in vitro ($\text{IC}_{50} = 14.7$ nM for 18 vs $\text{IC}_{50} = 8.04$ nM for 17, 3.75 nM for 19) and 4-fold less potent in vivo with respect to 17 and 19 ($\text{OD} = 1.56$ mg/kg for 18 vs $\text{OD} = 0.39$ mg/kg both for 17 and 19), exhibited a %T/C of 457, which was almost 3 times higher than that of 17 (%T/C = 174) and 19 (%T/C = 200). Compounds 17–19, although 2 orders of magnitude less cytotoxic than the nitrogen mustard derivative 7 with four pyrrole units, showed superior activity in vivo (%T/C = 174, 457, and 200 for 17–19, respectively, vs %T/C = 133 for 7).

As reported for other series of distamycin deriva-

tives,¹⁸ compounds 20–22, bearing the α -bromoacryloyl moiety, gave better results in terms of activity both in vitro and in vivo, in comparison to the corresponding derivatives 13–15, which possess the BAM function as the alkylating moiety. In fact compounds 20–22 were at least 10-fold more cytotoxic than 13–15 and, with the exception of 22, showed %T/C values which were higher than those reported for the benzoyl mustard counterparts.

In the same series 20–22, although the indole derivative 20 showed the same cytotoxicity as the benzofuran counterpart 22, the latter compound was 15-fold less potent in vivo and produced an increased survival time 2-fold higher than that for 20 ($\text{OD} = 0.78$ mg/kg, %T/C = 117 for 22 vs $\text{OD} = 12.5$ mg/kg, %T/C = 213 for 20). From the comparison of the compounds bearing the nitrogen mustard or α -bromoacryloyl unit as the alkylating moiety, it was found that derivatives 20 and 21 were more cytotoxic, but less potent in vivo, than the *N*-nitrogen mustard counterpart 17 and 18, since 22 was both less cytotoxic and potent than 19. The same compounds 20–22 maintained cytotoxicity substantially equivalent to that of the pyrrole 8 and pyrazole 10 analogues, but much higher with respect to the imidazole derivative 12.

A fairly marked correlation between antitumor activity and length of the polypyrrolic skeleton was observed for compounds 21 and 34, where the derivative 34 with two *N*-methylpyrrolic units was 5-fold less cytotoxic than the corresponding pyrrole homologue 21. This is in agreement with the hypothesis that DNA binding, which depends on the multiplicity of interactions between the pyrrolecarbamoyl units and AT-rich sequences of DNA, is crucial for cytotoxicity.¹⁹ Derivative 34, despite a potency 2-fold higher than that of 21 ($\text{OD} = 6.25$ vs 3.13 mg/kg), was slightly less effective than the latter in vivo (%T/C = 192 vs 133 for 21 and 34, respectively).

In a preliminary in vivo evaluation against the M5076 solid tumor, compound 21 was almost 4-fold less toxic than 18 ($\text{OD} = 0.93$ mg/kg for 18 vs $\text{OD} = 3.13$ mg/kg for 21), with an increased %T/C slightly lower (%T/C = 124 for 18 vs %T/C = 154 for 21), while both compounds showed the same trend of inhibition of tumor growth (%TI = 92 for 18 vs %TI = 96 for 21).

In Table 2 the resistance index (RI) values of 1–22, 34, and 64 on leukemia cell lines resistant to doxoru-

Table 2. Cytotoxicity and Resistance Index (RI) of Distamycin Derivatives against L1210 Leukemia Cells Resistant to Doxorubicin (DX) and Tallimustine^a

compd	L1210/DX		L1210/tallimustine	
	IC ₅₀ (μ M \pm SE)	RI	IC ₅₀ (μ M \pm SE)	RI
distamycin A ³	459 \pm 50.1	45.6	142 \pm 13.4	14.1
tallimustine (2) ³	2.6 \pm 0.2	38.5	0.55 \pm 0.06	8
3 ⁴	nd	nd	nd	nd
4 ³	5.4 \pm 1	68.0	12.4 \pm 2.5	156
5 ³	78.1 \pm 6.3	175.4	75.6 \pm 42.3	169.9
6 ³	nd	nd	1.24 \pm 0.3	65.4
7 ⁴	nd	nd	nd	nd
8 ³	0.024 \pm 0.0002	3.8	0.17 \pm 0.013	27.0
9	11.3 \pm 1.2	326.2	10.4 \pm 1.2	302.3
10	0.093 \pm 0.017	7	0.55 \pm 0.13	41.4
11	4.7 \pm 1.2	2	5 \pm 0.082	2.1
12	0.73 \pm 0.069	15.5	0.65 \pm 0.14	13.9
13	0.54 \pm 0.039	5.7	0.88 \pm 0.039	9.2
14	0.42 \pm 0.041	6.8	1.2 \pm 0.13	19
15	0.34 \pm 0.01	7.8	12.9 \pm 0.58	295.8
16	3.8 \pm 0.59	2.7	nd	nd
17	0.059 \pm 0.0007	7.3	0.093 \pm 0.00021	11.6
18	0.11 \pm 0.01	7.4	0.078 \pm 0.002	5.3
19	0.07 \pm 0.01	18.7	0.085 \pm 0.008	22.8
20	0.036 \pm 0.0056	8.7	0.14 \pm 0.03	34.9
21	0.043 \pm 0.015	18	0.15 \pm 0.036	64.8
22	0.039 \pm 0.002	6.4	0.21 \pm 0.019	33.8
34	0.041 \pm 0.0009	2.7	0.15 \pm 0.076	10.1
64 ¹⁶	0.13 \pm 0.10	17.6	0.11 \pm 0.023	8.00

^a IC₅₀ = 50% inhibitory concentration as the mean \pm SE from dose-response curves of at least three experiments; RI (resistance index) = ratio between IC₅₀ values on resistant cells and sensitive cells.

bicin (Dx) and tallimustine are reported. The results obtained show that, for the newly synthesized compounds 13–22 and 34, only 13, 17, 18, and 34 display low RI values for leukemia cell lines resistant to doxorubicin and tallimustine. For each new derivative, with the exception of 18, which was more active in the tallimustine-resistant L1210 leukemia line, it was interesting to note that the RI values in L1210/tallimustine cells were at least 2-fold higher than that reported in L1210/DX cells, showing that neither the alkylating moiety nor the benzoheterocycle were involved in the resistance mechanism.

It is also interesting to note that in the comparison between compounds 13 and 14, 17 and 18, 20 and 21 which bear the same alkylating moieties, N-methylation of the indole nucleus had an important effect only for the %T/C value of 18. Finally, nitrogen mustard and α -bromoacryloyl derivatives showed comparable cytotoxicity against L1210 murine leukemia cells, superior to that reported for BAM derivatives with the same oligopeptidic frame.

Arrested Polymerase Chain Reaction (PCR) and Direct DNA Fragmentation Assay. To determine the influence of the alkylating group and the oligopeptide skeletons on sequence selectivity, two series of derivatives, 13–16 and 17–19, were compared, employing molecular studies, such as arrested PCR and a direct DNA fragmentation assay.

First, we determined the effect of compounds 13–16 and 17–19 on PCR-mediated amplification of two genomic sequences: one rich in A+T, the other rich in G+C. The human estrogen receptor (ER) gene (A+T/G+C = 3.46) and the human Ha-ras oncogene (A+T/G+C = 0.37) were chosen as model systems, as they are suitable for use in determining sequence-selective bind-

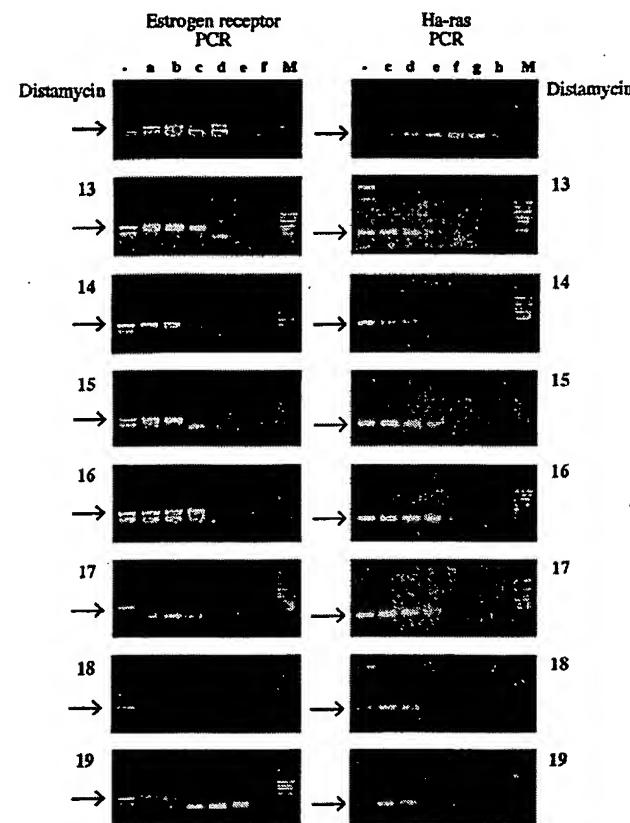


Figure 1. Effects of distamycin and distamycin analogues 13–19 on PCR-mediated amplification of estrogen receptor (ER; left side of the panel) and Ha-ras (right side of the panel) genomic sequences. For PCR-mediated amplification the target DNA was 20 ng of human genomic DNA or 2 ng of pBLCAT1-ERCAT8; PCR buffer, Taq DNA polymerase, and the four dNTPs were added as elsewhere described.²¹ Before PCR, target DNA was incubated in the absence (–) or presence (a–h) of DNA-binding compounds (a = 1 μ M, b = 2 μ M, c = 5 μ M, d = 10 μ M, e = 20 μ M, f = 50 μ M, g = 100 μ M, h = 200 μ M) for 10 min. Conditions of Ha-ras PCR were: denaturation, 92 °C, 45 s; annealing, 62 °C, 45 s; elongation, 72 °C, 30 s (25 cycles). Conditions of ER PCR were: denaturation, 92 °C, 45 s; annealing, 55 °C, 1 min; elongation, 72 °C, 1 min (25 cycles). Amplified DNA was analyzed by electrophoresis on 2.5% agarose, 0.5 mg/mL ethidium bromide. Specific ER and Ha-ras PCR products are arrowed. Specificity of ER and Ha-ras PCR products was confirmed by Southern blotting and hybridization to specific ³²P-labeled probes as well as isolation of the arrowed PCR products and direct sequencing (ref 22 and data not shown).

ing of DNA-binding compounds when amplified by PCR.^{20,21} We have recently published the nucleotide sequence of a 3.2-kb genomic region located upstream of the estrogen receptor sequence²² originally designated exon-1 and demonstrated that this region contains A+T-rich sequences recognized by distamycin A and distamycin analogues.²³ The human Ha-ras oncogene sequence, on the other hand, is G+C-rich and therefore interacts with low efficiency with distamycin, being, on the contrary, efficiently recognized by G+C-selective binders, such as chromomycin and mithramycin.²⁰ Figure 1 shows a representative example of the PCR experiment performed using distamycin A (1) and distamycin analogues; Table 3 shows all the results obtained, expressed as the inhibitory activity (IC₅₀) of

Table 3. Inhibitory Activity (IC_{50}) of Distamycin Derivatives on the Generation of ER and Ha-ras PCR Products

compd	PCR IC_{50}^a (μ M)		PCR IC_{50}^a (μ M)			
	ER	Ha-ras	compd	ER	Ha-ras	
distamycin A	20	200	16	10	50	
13	7.5	15	17	1	20	
14	5	15	18	1	20	
15	3	20	19	3	15	

^a Inhibitory concentration (mM) necessary to obtain 50% inhibition of generation of PCR products of human Ha-ras and ER sequences.

distamycin derivatives and the generation of ER and Ha-ras PCR products.

In agreement with already published reports,^{20,21} distamycin A (1) inhibits ER PCR (Figure 1, left side of the panel) but exhibits lower capacity to inhibit Ha-ras PCR (Figure 1, right side of the panel), thus confirming a selective binding of distamycin to A+T-rich gene sequences. IC_{50} of distamycin was found to be 20 μ M in the case of ER PCR and over 200 μ M in the case of Ha-ras PCR. By contrast, compounds 13–15 and 17–19 are effective inhibitors of both ER and Ha-ras PCR (Figure 1), maintaining however sequence selectivity for ER A+T-rich gene sequences (see Table 3 for semiquantitative analysis). These data suggest that sequence-selective binding typical of distamycin is to some extent maintained by these compounds. As reported in Table 3, when compounds 13–15 are compared to the corresponding 17–19, no major differences were found with respect to inhibitory activity on the generation of Ha-ras PCR products. By contrast, compounds 17–19 were found to be consistently more active than the corresponding 13–15 in inhibiting the generation of ER PCR products (Figure 1 and Table 3).

To further investigate sequence selectivity of compounds 13–16 and 17–19, arrested PCR fragments generated using a ³²P-labeled ER PCR primer were analyzed by gel electrophoresis and the sites of arrest identified. The results obtained are shown in Figure 2 and demonstrate that the sequence selectivity of compounds 13–16 and 17–19 is readily observed. The nucleotide sequences of the sites of arrest are also shown in Table 4.

Compounds 15 and 19 were the most active compounds (inhibition of generation of full-length PCR product was obtained at 2 μ M concentration). The analysis of the nucleotide sequences corresponding to the sites of arrest of the PCR demonstrate that the arrested sites (5'-AGTTAAAA-3' and 5'-TAAT-3') were found when the arrested PCR was performed in the presence of all the compounds tested. The arrested site 5'-TTAACTT-3' was preferentially found in the presence of compounds 14 and 15, also being clearly detectable in the presence of compounds 13 and 17–19. The arrested site 5'-ATGTGTGTGTA-3' was preferentially found in the presence of compounds 18 and 19.

A first conclusion gathered from the data shown in Figure 2 is that both alkylating groups and oligopeptide skeletons play a crucial role in determining the sequence selectivity of these compounds. Direct DNA fragmentation assays (Figure 3) confirmed these conclusions. In particular, it should be noted that compounds 17–19 are more active in this assay than compounds 13–16. In addition, comparative analysis of the lanes corre-

sponding to compounds 17–19 shows that a different pattern of fragmentation is generated after the direct DNA fragmentation assay (see the fragments corresponding to the site 5'-TATGTACGTGTGC-3'), suggesting differences of binding activity of compounds 17–19. Further experiments employing other genomic experimental systems exhibiting similar GT-rich nucleotide sequences will be necessary in order to determine whether this observation could be generalized. In any case, it should be emphasized that major arrested sites of PCRs (Figure 2) extensively overlap with the nucleotide sequences corresponding to the fragments generated when the direct DNA fragmentation assay was performed (Figure 3).

Furthermore, it should be noted that a good relationship exists between activity in the arrested PCR assay (Table 3) and in vitro cytotoxicity (Table 1). For instance, compounds 17–19 were found to be more active than compounds 13–15 in both the in vitro cytotoxicity tests and the arrested PCR assays. Accordingly, compound 19 was found to be more active than 17 and 18 in both the in vitro cytotoxicity tests and the arrested PCR and direct DNA fragmentation assays. Compound 16 was consistently found to be less active than compounds 13–15.

Conclusions

All the novel compounds, with the exception of compound 16, showed in vitro activity against L1210 murine leukemia cell line comparable to or better than that of tallimustine. Compounds in which the nitrogen mustard and the α -bromoacryloyl moieties are directly linked to the benzoheterocyclic ring showed very high equipotent activities, ranging from 2 to 14 nM, while benzoyl nitrogen mustard derivatives of benzoheterocycles showed lower activities, with one compound (16) of this cluster being the only derivative devoid of significant activity. Despite the fact that in vivo anti-leukemic activity was found to be generally poorly correlated with cytotoxicity, the exceptional antileukemic activity of 18 is noteworthy. Therefore, compound 18 has been selected for further extensive evaluation on murine solid tumors and human xenograft. The data obtained in arrested PCR and direct DNA fragmentation assays suggest that both the alkylating groups and the oligopeptide frames play a crucial role in determining the sequence selectivity of these compounds. In conclusion, the results presented in this paper suggest that the synthesis of various derivatives of distamycin A may be a useful approach to cancer chemotherapy, leading to the production of structurally related compounds exhibiting significant antitumor activity.

Experimental Section

Chemical Materials and Methods. General Procedure. All reactions were carried out under argon atmosphere, unless otherwise described. Standard syringe techniques were applied for transferring anhydrous solvents. Reaction courses and product mixtures were routinely monitored by thin-layer chromatography on silica gel (precoated F₂₅₄ Merck plates), the spots were examined with UV light and visualized with aqueous KMnO₄. ¹H NMR spectra were recorded in the given solvent with a Bruker AC 200 spectrometer. Chemical shifts are reported as δ values in ppm. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet), t (triplet), br (broad), and m (multiplet). Melting

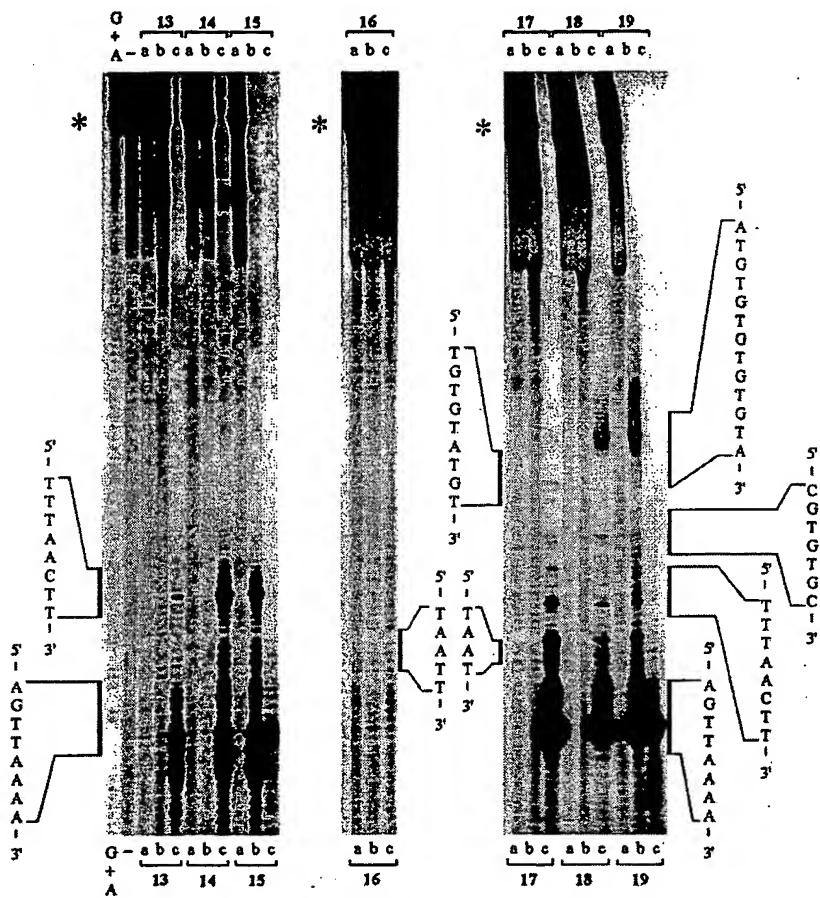


Figure 2. Arrested PCR: analysis of arrested sites. In this experiment, ER target DNA was prepared by PCR. After incubation in the absence (−) or presence of (a) 0.5, (b) 2, and (c) 10 μ M DNA-binding compounds, a further 10 cycles of PCR were performed with a 32 P-labeled ER reverse PCR primer. After PCR, the samples were heated at 92 °C for 5 min and layered onto a sequencing gel. The nucleotide sequences of the sites of PCR arrest are indicated. Asterisks indicate full-sized PCR products. At these compound concentrations distamycin A generates no appreciable sites of ER PCR (data not shown).

Table 4. Arrested PCR: Sequence Recognition by Distamycin Derivatives

compd	nucleotide sequence
18 and 19	5'-ATCTGTGTGTGA-3'
17	5'-TCTCTATGT-3'
17-19	5'-CCTGTGC-3'
13-15 and 17-19	5'-TTAACCTT-3'
13-19	5'-TAATT-3'
13-19	5'-CACTTAAAA-3'

points (mp) were determined using a Buchi-Tottoli apparatus and are uncorrected. All products reported showed 1 H NMR spectra in agreement with the assigned structures. Mass spectra were recorded on a Nermag R10,10C spectrometer. Elemental analyses, conducted by the Microanalytical Laboratory, Chemistry Department, University of Ferrara, were within 0.4% of the theoretical values calculated for C, H, Br, Cl, and N. Column chromatography was carried out Merck silica gel (230–240 mesh). All compounds obtained commercially were used without further purification. Organic solutions were dried over anhydrous MgSO_4 . Methanol was distilled from magnesium turnings, dioxane was distilled from calcium hydride, and anhydrous DMF was distilled from calcium chloride and stored over molecular sieves (3 Å). In high-pressure hydrogenation experiments, a Parr shaker on a high-pressure autoclave was used.

General Procedure A for the Synthesis of 38 and 39. A solution of the appropriate nitro ester (2 mmol) in 10 mL of a mixture of MeOH/dioxane (1:1, v/v) was hydrogenated over 50 mg of 10% Pd/C at 60 psi for 5 h. The catalyst was removed by filtration; the filtrate was concentrated to give a green oil

which was precipitated from EtOAc/petroleum ether and used without purification for the next step.

General Procedure B for the Synthesis of BAM Esters 41–44. A solution of *p*-[*N,N*-bis(2-chloroethyl)amino]benzoyl chloride (40) (310 mg, 1.1 mmol) in anhydrous CH_2Cl_2 (5 mL) was added dropwise in small portions to a mixture of amino ester 36–39 (1 mmol) and triethylamine (0.14 mL, 1 mmol) in anhydrous CH_2Cl_2 (5 mL) cooled to 0 °C. The reaction mixture was stirred at room temperature for 18 h and concentrated under reduced pressure yielding a brown solid, which was dissolved in EtOAc (20 mL) and washed with 2 N hydrochloric acid (2 × 10 mL). The organic layer was dried (Na_2SO_4) and concentrated and the resulting residue precipitated from EtOAc/petroleum ether to give 41–44.

General Procedure C for the Synthesis of 23–26. A well-stirred solution of 41–44 (1 mmol) in dioxane (4 mL) was treated with 2 N KOH in water (1 mL) and stirred at room temperature for 3 h. The clear solution was evaporated to remove dioxane, diluted with water (5 mL), cooled on an ice–water bath and acidified with 2 N hydrochloric acid to pH 2. The aqueous suspension was extracted with EtOAc (2 × 10 mL) and the organic layers were combined, dried (Na_2SO_4) and concentrated. The resulting residue was precipitated from EtOAc–hexane to give the product 23–26.

General Procedure D for the Synthesis of Intermediates 45–47. To a solution of amino ester 36–38 (1 mmol) in MeOH (10 mL) cooled at –10 °C was added cold ethylene oxide (2.5 mL). The reaction flask was sealed and allowed to reach room temperature overnight. Methanol and excess of ethylene oxide were removed by evaporation and the crude residue purified by flash chromatography on silica gel.

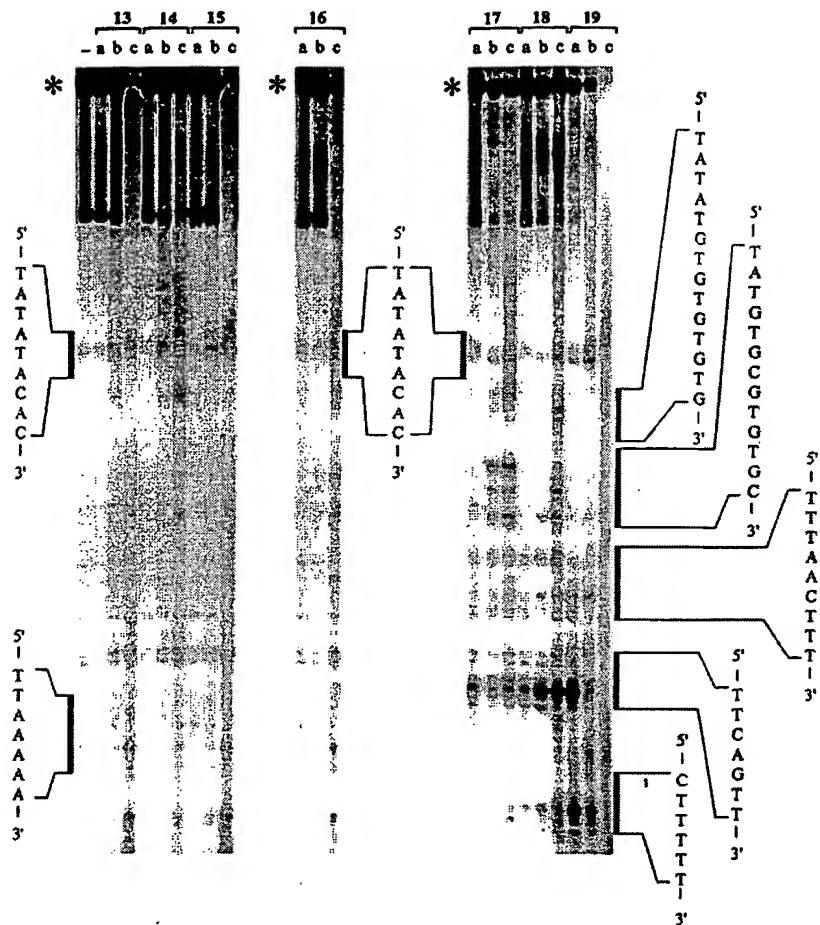


Figure 3. DNA cross-linking assay. In this experiment a ^{32}P -labeled ER PCR product was produced using a ^{32}P -labeled ER reverse PCR primer. After production of the ER PCR probe, an aliquot was incubated in 50 μL in the absence (–) or presence of (a) 0.5, (b) 2, and (c) 10 μM DNA-binding compounds. After 5 h incubation at room temperature the samples were heated at 90 °C for 30 min, ethanol-precipitated, and analyzed by electrophoresis on a sequencing gel. The nucleotide sequences corresponding to DNA fragmentation are indicated. Asterisks indicate full-sized PCR products.

General Procedure E for the Synthesis of the Nitrogen Mustard Esters 48–50. The compound 45–47 (1 mmol) was cooled in an ice bath and 2 mL of phosphorus oxychloride (20 mmol, 2 mL) was added dropwise. The solution was heated at 100 °C for 1 h, the solvent evaporated under vacuo, and then the residue dissolved in EtOAc (8 mL) and washed with water (3 mL). The organic phase was dried (Na_2SO_4) and concentrated and the crude product purified by flash chromatography on silica gel.

General Procedure F for the Synthesis of Nitrogen Mustard Carboxylic Acids 27–29. The nitrogen mustard 48–50 (1 mmol) was dissolved in 36% hydrochloric acid (4 mL) and heated at 100 °C for 3 h. The solution was cooled at room temperature, diluted with water (10 mL) and extracted with EtOAc (2 \times 20 mL). The recombined organic phases were dried (Na_2SO_4) and evaporated in vacuo and the residue was purified by flash chromatography on silica gel.

General Procedure G for the Synthesis of Nitro *tert*-Butyl Esters 55 and 56. A solution of nitro acid 52–53 (5 mmol) in dry DMF (10 mL) cooled at 0 °C was treated with CDI (1.1 g, 6.5 mmol). After 1 h at room temperature, *tert*-butyl alcohol (1 mL) and DBU (0.9 mL) were added. The mixture was stirred for 2 h and poured in cooled water (50 mL) and the resulting crystalline solid collected and washed with water, affording the desired nitro *tert*-butyl ester 55–56.

General Procedure H for the Reduction of Nitro *tert*-Butyl Esters 54 and 56. The nitro *tert*-butyl ester 54–56 (5 mmol) was reduced over 10% Pd/C (100 mg) in a mixture of dioxane (10 mL) and methanol (10 mL) at room temperature

under 55 psi pressure. After reduction, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue, after purification by flash chromatography, was dissolved in EtOAc and precipitated with an excess of petroleum ether.

General Procedure I for the Synthesis of Bromoacryl-amido *tert*-Butyl Esters 60–62. To a solution of α -bromoacrylic acid (600 mg, 4 mmol) in dry DMF (5 mL) was added a solution of EDC (383 mg, 2 mmol) in dry DMF (5 mL) slowly. After 1 h, the solution obtained was added to a solution of amino *tert*-butyl ester 57–59 (2 mmol) in dry DMF (5 mL). The reaction was stirred at room temperature for 18 h and then concentrated under reduced pressure. The residue was dissolved with a mixture of EtOAc (10 mL) and water (5 mL), the organic phase dried over Na_2SO_4 and evaporated to dryness in vacuo, and the resulting crude residue purified by flash chromatography.

General Procedure J for the Synthesis of 30–32. A solution of the ester 60–62 (1 mmol) in CF_3COOH (1 mL) was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the resulting residue was suspended in CH_2Cl_2 (3 mL), collected by filtration and triturated with ethyl ether (5 mL) to obtain 30–32.

General Procedure K for the Synthesis of Compounds 13–22. A solution of *N*-deformyldistamycin A dihydrochloride (33) (263 mg, 0.5 mmol) in dry DMF (5 mL) was cooled to 5 °C and *N,N*-diisopropylethylamine (86 μL , 0.5 mmol) was added. After 10 min, the acid 23–32 (0.6 mmol, 1.2 equiv) and then EDC (192 mg, 1 mmol) were added. The reaction mixture was stirred at room temperature for 18 h, then 2 N hydro-

chloric acid was added until $\text{pH} = 4$. The solvent was evaporated in vacuo and the crude residue purified by flash chromatography to yield an oil, which was precipitated from MeOH/diethyl ether.

Biological Testing in Vitro Using L1210 Cells. The murine lymphocytic L1210 leukemia cell line was obtained from the American Type Culture Collection (ATCC). All the tested compounds were dissolved in DMSO at 1 mg/mL immediately before use and diluted in medium before addition to the cells. The murine lymphocytic leukemia cells L1210, L1210/DX and L1210/tallimustine were grown in vitro as a stationary suspension culture in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (Flow, Irvine, U.K.), 2 mM L-glutamine (GIBCO), 10 mM β -mercaptoethanol, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. To determine survival after compound exposure, exponentially growing L1210 cells were continuously exposed to various concentrations of compounds for 48 h, after which the cytotoxic activity of the compounds was evaluated by counting surviving cells using an electronic cell counter ZBI (Coulter Counter Electronics, Hialeah, FL). The cytotoxic activity of the compounds was calculated from dose-response curves and expressed as IC_{50} (concentration of test compound to reduce the cell number to 50% of that obtained with untreated control cells). All experiments were repeated at least twice. For each compound concentration, duplicate cultures were used. Vehicle or solvent controls were run with each experiment.

Biological Tests in Vivo. DBA/2N female mice were used for implanting with the murine L1210 leukemia. For experiments with leukemia CD2F1 female mice were used. C57B16 female mice were used for implanting with the murine reticulosarcoma M5076. Charles River Italia (Calco, Como, Italy) supplied all mice. Mice were 8–17 weeks and weighed 20–22 g at the time of tumor implantation. Animal health was monitored by serological testing; the animals were free of infectious pathogens, including mouse hepatitis virus, Sendai virus and *Mycoplasma pulmonis*, during the course of experimentation. All compound solutions were prepared immediately before use and given intravenously (iv) in a volume of 10 mL/kg of body weight. The vehicle used in preparation of solutions consisted of 10% Tween 80 and 90% saline.

L1210 murine leukemia (originally obtained from the National Cancer Institute, Frederick, MD) was maintained in vivo by continuous ip passage (10^6 cells/mouse), for experiments 10^5 cells/mouse, 10 mice/group, were injected ip or iv. Compounds were administered iv or ip at day 1 after tumor cell injections. A dose-response was determined in all experiments. Toxicity was evaluated on the basis of the gross autopsy findings and the weight loss, mainly in terms of reduction of spleen and liver size.

M5076 reticulosarcoma was maintained in vivo by im serial transplantation. For experiments, 5×10^5 cells were injected im in the flank of C57B16 female mice. Animals were 8–10 weeks old at the beginning of the experiments. Compounds were administered iv at day 3, 7, and 11 after tumor implantation. Survival time of mice and tumor growth were calculated and activity was expressed in terms of %T/C and %TI. %T/C = median survival time treated group vs median survival time untreated group $\times 100$. The survival time of control mice injected iv with L1210 is 6 days, while for ip injected mice it is 8–10 days. %TI = % inhibition of tumor growth with respect to control. Compounds were considered active if the %T/C value was ≥ 125 .

Target DNA, Oligonucleotide Primers, and Arrested PCR. The sequences of ER^{24,25} and Ha-ras²⁶ primers used for PCR²⁷ were the following: ER-forward, 5'-GACGCATGATAT-ACTTCACC-3'; ER-reverse, 5'-GCAGAACATAATCCAGATG-3'; Ha-ras forward, 5'-AGACGTGCCTGTTG GACATC-3'; Ha-ras reverse, 5'-CGCATGTACTGGTCCCCAT-3'. Taq DNA polymerase was purchased from FINNZYMES OY (Espoo, Finland) and added at 2.5 U/25 μL final concentration. When using FINNZYMES Taq DNA polymerase, distamycin A was found to inhibit ER amplification when present at 2–10 μM final concentrations, depending upon the type of target DNA

(genomic DNA, recombinant plasmid, PCR product). For PCR-mediated amplification the target DNA was 20 ng of genomic DNA or 2 ng of pBLCAT1-ERCAT8; PCR buffer, Taq DNA polymerase and the four dNTPs were added as elsewhere described.²¹ Conditions of PCRs were: denaturation, 92 °C, 1 min; annealing, 55 °C (ER) or 62 °C (Ha-ras), 1 min; elongation, 72 °C, 1 min (25 cycles). The effects of DNA-binding drugs were analyzed after incubating target DNA at room temperature, for 5 min, with increasing amounts of the compounds followed by PCR. Amplified DNA was analyzed by electrophoresis on 2% agarose gels. For sequence analysis of arrested PCR we first prepared ER target DNA by PCR. After incubation for 1 h at 37 °C with the distamycin analogues, samples were heated at 90 °C for 5 min and PCR was performed using the ³²P-labeled ER reverse PCR primer (5'-GCAGAACATAATCCAGATG-3'). After PCR, each reaction was resuspended in 5 μL of loading dye (0.1% xylene-cyanol, 0.1% bromophenol blue, 0.1 M NaOH:formamide 1:2) and electrophoresed through a sequencing gel as described.²¹

Direct DNA Fragmentation Assay. A ³²P-labeled ER PCR product was produced using a ³²P-labeled ER reverse PCR primer (5'-GCAGAACATAATCCAGATG-3'). After production of the ER PCR probe, an aliquot was incubated in 50 μL of 0.1 \times SSC in the presence of DNA-binding drugs. After 5-h incubation at room temperature the samples were heated at 90 °C for 30 min and ethanol was precipitated. Each reaction was resuspended in 5 μL of loading dye (0.1% xylene-cyanol, 0.1% bromophenol blue, 0.1 M NaOH:formamide 1:2) and electrophoresed through a sequencing gel as described.²¹

Acknowledgment. We thank Pharmacia & Upjohn and Ministero Università e Ricerca Scientifica (MURST) (60%) for generous financial support of this work. R.G. is supported by ISS (AIDS-98), PRIN-98, P.F. Biotecnologie, and Progetto per la Ricerca Finalizzata 1998 (Ministero della Sanità). We thank Dr. Alessia Felloni for performing the arrested PCR experiments reported herein.

Supporting Information Available: Experimental procedures and ¹H NMR spectra for compounds 13–32, 34, and 38–62. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JM9911229